*Kidney International, Vol. 56 (1999), pp. 1674–1682*

# Autosomal recessive distal renal tubular acidosis associated with Southeast Asian ovalocytosis

**Somkiat Vasuvattakul, Pa-thai Yenchitsomanus, Prayong Vachuanichsanong, Peti Thuwajit, Charoen Kaitwatcharachai, Vichai Laosombat, Prida Malasit, Prapon Wilairat, and Sumalee Nimmannit**

*Renal Division and Medical Molecular Biology Unit, Siriraj Hospital, Mahidol University; Songklanakarin Hospital, Prince of Songkla University, Songkla; and Department of Biochemistry, Faculty of Science, Mahidol University, Bangkok, Thailand*

**Autosomal recessive distal renal tubular acidosis associated with Southeast Asian ovalocytosis.**

*Background.* A defect in the anion exchanger 1 (AE1) of the basolateral membrane of type A intercalated cells in the renal collecting duct may result in a failure to maintain a cell-to-lumen $H^+$ gradient, leading to distal renal tubular acidosis (dRTA). Thus, dRTA may occur in Southeast Asian ovalocytosis (SAO), a common *AE1* gene abnormality observed in Southeast Asia and Melanesia. Our study investigated whether or not this renal acidification defect exists in individuals with SAO.

*Methods.* Short and three-day $NH_4Cl$ loading tests were performed in 20 individuals with SAO and in two subjects, including their families, with both SAO and dRTA. Mutations of *AE1* gene in individuals with SAO and members of the two families were also studied.

*Results.* Renal acidification in the 20 individuals with SAO and in the parents of the two families was normal. However, the two clinically affected individuals with SAO and dRTA had compound heterozygosity of 27 bp deletion in exon 11 and missense mutation G701D resulting from a CGG→CAG substitution in exon 17 of the *AE1* gene. Red cells of the two subjects with dRTA and SAO and the family members with SAO showed an approximate 40% reduction in sulfate influx with normal 4,4′-di-isothiocyanato-stilbene-2,2′-disulfonic acid sensitivity and pH dependence.

*Conclusion.* These findings suggest that compound heterozygosity of abnormal *AE1* genes causes autosomal recessive dRTA in SAO.

Southeast Asian ovalocytosis (SAO) is a hereditary condition that is widespread in parts of Southeast Asia and Melanesia. It has been shown that SAO results from a mutation in the red cell membrane band 3 or the anionic ($HCO_3^-/Cl^-$) exchanger 1 (AE1) [1, 2]. The N-terminal fragment of the abnormal band 3 migrates slower than normal in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) [3]. Sequencing of the abnormal erythrocyte *AE1* gene in SAO showed that it contained two linked mutations: a deletion of codons 400 to 408 in the boundary of cytoplasmic and membrane domains and a point mutation in the first base of codon 56 (K56E), the Memphis I polymorphism [4].

Anion exchanger 1 is also found in the basolateral membrane of the type A intercalated cells of renal collecting ducts, which are involved in $H^+$ secretion [5, 6]. Studies of human kidneys have indicated that, although the protein in the basolateral membrane of type A intercalated cells is reactive toward monoclonal antibodies to the membrane transport domain of AE1 [6, 7], several antibodies to the cytoplasmic domain of AE1 are unreactive [6], which is consistent with renal AE1 being truncated at the $NH_2$ terminus [8]. A promoter that gives rise to these kidney transcripts is present in erythroid intron 3 of the human *AE1* gene [8, 9].

A defect in AE1 of the basolateral membrane of type A intercalated cells of the collecting duct may result in a failure to establish or maintain a cell-to-lumen $H^+$ gradient, and leads to distal renal tubular acidosis (dRTA) [5, 10]. Three studies and a review have shown that mutations of the *AE1* gene affect renal acidification [11–14]. There are at least two reports indicating an association between dRTA and hereditary elliptocytosis [15, 16], which is uncommon among Caucasians, but a related condition, SAO, is widespread in parts of Southeast Asia, with a prevalence reaching 30% in certain ethnic groups [17].

To examine the possibility that a defect in renal acidification may be associated with subjects with SAO, the renal acidification function and a detailed characterization of the *AE1* gene were studied in SAO individuals and members of two unrelated families with dRTA and

**Key words:** band 3 protein, anion exchanger 1, *AE1* gene, DNA sequencing, renal acidification.

Received for publication November 17, 1998
and in revised form June 15, 1999
Accepted for publication June 16, 1999

SAO. In this report, we describe a novel compound heterozygosity of mutated *AE1* genes in the subjects with SAO and dRTA, and an autosomal recessive mode of inheritance of the abnormal genes associated with the two combined defects.

## METHODS

### Subjects

The study population consisted of 20 individuals with SAO, two unrelated subjects with SAO and dRTA (as defined by a low rate of $NH_4^+$ excretion and an inability to lower the urine pH below 5.5 in the presence of systemic acidosis, $HCO_3^- < 20$ mEq/liter) and their family members and 11 individuals with normal red blood cell morphology living in the same region as the control subjects. All subjects were placed on a normal diet, and medications were terminated one week prior to the study. This investigation was approved by the Human Ethics Committee of the Prince of Songkla University, Thailand.

### Clinical studies

Renal acidification was studied using a short acid loading by administration of 0.1 g/kg $NH_4Cl$, as previously described by Wrong and Davies [18], in 10 individuals with SAO, the two family members and seven normal control subjects; simultaneously, a chronic acid loading was achieved by administration of 0.1 g/kg/day $NH_4Cl$ for three days with diuresis on the fourth day induced by furosemide (20 mg p.o.) [19] in another 10 individuals with SAO and four normal control subjects. To achieve urinary osmolality >800 mOsm/kg $H_2O$, intranasal 1-deamino-D-arginine vasopressin (DDAVP) was given after 16 hours of water deprivation [20].

Venous blood pH was measured using blood gas analyzer (model 178, Corning). The concentration of bicarbonate in plasma was calculated using the pK value of 6.10 and a solubility factor of 0.0301 [21, 22]. Analytical methods for determination of $NH_4^+$, sodium, potassium, chloride, creatinine, and osmolality were as previously described [23].

The clearance of creatinine provided an approximation of the glomerular filtration rate [24]. The transtubular K concentration gradient (TTKG) was calculated to reflect the driving force for K secretion [25].

Values were reported as mean ± SEM, and comparisons between groups were made by analysis of variance (ANOVA).

### DNA analysis

*Polymerase chain reaction primers.* The sequence of *AE1* gene was retrieved from Entrez database (GenBank, NCBI). Nineteen pairs of polymerase chain reaction (PCR) primers were designed for amplifications of two overlapping regions in intron 3 (one for the potential kidney promoter and another for the possible 5′ sequence of transcript expressed in kidney) and 17 regions in exons 4 to 20 of the *AE1* gene (Table 1). The primers for each exon would anneal to sequences in introns, flanking both sides of the exon. The sizes of PCR products obtained from amplifications using these primers were usually less than 400 bp, except those for the two regions in intron 3. These primers were synthesized by BioService Unit of the National Center for Genetic Engineering and Biotechnology (Biotec, Bangkok, Thailand).

*DNA samples.* Leukocyte genomic DNAs were prepared from 10 ml ethylenediaminetetraacetic acid (EDTA) blood samples by standard DNA extraction method, which consisted of steps of proteinase K digestion, phenol-chloroform extractions, and ethanol precipitation [26]. DNA samples were finally dissolved in sterile distilled water, and their amounts were estimated from absorbances measured by ultraviolet-visible spectrophotometer at wavelength 260. A small part of stock DNA sample was diluted to 50 ng/μl for using in PCR.

*Polymerase chain reaction.* Polymerase chain reaction was performed by mixing 125 ng DNA sample, 2.5 μl of 10 × buffer (Perkin-Elmer Cetus, Norwalk, CT, USA), 1.5 μl of 25 mM $MgCl_2$, 2.5 μl of 2 mM dNTP mix, 12.5 pmol each of forward (L) and reverse (R) primers, and 0.25 units of *Taq* polymerase (Perkin-Elmer Cetus) in a total volume of 25 μl. The reaction mixture was overlaid with one drop of mineral oil, and amplification was performed for 35 cycles in Thermal Cycler 480 (Perkin-Elmer Cetus). Each cycle was comprised of denaturation at 94°C for one minute (5 min for the first cycle), annealing at 58 to 70°C (depending on pair of primers; Table 1) for one minute, and extension at 72°C for one minute (5 min for the final cycle). After amplifications, PCR products were examined by running on 2% agarose gel electrophoresis and ethidium bromide staining.

*Single strand conformational polymorphism (SSCP).* Two microliters of the PCR product were mixed with 8 μl of sample running buffer (containing 95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol, 20 mM EDTA, and 10 mM NaOH). The mixture was heated at 95°C for 10 minutes to denature DNA into single strands and was then cooled on ice for five minutes before loading onto nondenaturing polyacrylamide gel. The polyacrylamide gel with the size of 90 × 80 mm and thickness of 1 mm contained 10% acrylamide:bis-acrylamide (49:1). Electrophoresis was run in 1 × TBE buffer at 20 mA for two to six hours at room temperature. Double-stranded DNA might also be run by mixing 2 μl of the PCR product with 8 μl sample running buffer (without 10 mM NaOH) and loading onto the same gel without heating.

After electrophoresis, the gel was fixed with 40% methanol for 10 minutes, soaked in 160 mM $HNO_3$ for

**Table 1.** Oligonucleotide primers for amplifications of the *AE1* gene

| Exon | PCR primer sequence | Position | Annealing temp °C | Product size bp |
|---|---|---|---|---|
| Intron 3.1 | 5′-CAGTTTGGGACAAGGGCGTG-3′ | 6995–7014 | 67 | 491 |
| | 5′-TGATGAAGTGAAGGGACCTCTCC-3′ | 7463–7485 | | |
| Intron 3.2 | 5′-TGGGAGGAGAGAAGGGAGTCTG-3′ | 7364–7383 | 67 | 402 |
| | 5′-CGGTGTCGTGAGCTGAAAACC-3′ | 7745–7765 | | |
| Exon 4 | 5′-GTCTCTGAGGCTCACAGTGGATG-3′ | 7673–7695 | 63 | 226 |
| | 5′-ATCCCCTTGCTCCTCTCTTCC-3′ | 7878–7898 | | |
| Exon 5 | 5′-TGAGCACCCACTATGCCCTG-3′ | 8522–8541 | 63 | 299 |
| | 5′-CAGCACCCCACAACAATCCTC-3′ | 8800–8820 | | |
| Exon 6 | 5′-AGATGAGGATTGTTGTGGGGTG-3′ | 8796–8817 | 63 | 262 |
| | 5′-CAAGTGGGCTGGGGAAGTG-3′ | 9039–9057 | | |
| Exon 7 | 5′-CACCACTGATAGCTCAGCCTGAAC-3′ | 9407–9430 | 60 | 243 |
| | 5′-TGAGAAAGCTCTCTCCTTGCCC-3′ | 9628–9649 | | |
| Exon 8 | 5′-GAGAATGGGAAGGGGAGGATG-3′ | 9739–9760 | 60 | 244 |
| | 5′-GGTCCAGGCTGAGGGAAAGAC-3′ | 9963–9983 | | |
| Exon 9 | 5′-TCTTCAGCACACCCACCCTG-3′ | 9998–10017 | 60 | 299 |
| | 5′-TCAGCCACCATGCAGGTCC-3′ | 10278–10296 | | |
| Exon 10 | 5′-TCCTTTCCCTCCGCAGGTC-3′ | 10726–10744 | 58 | 332 |
| | 5′-ACAGAGGCTACGCTGAGGTGTC-3′ | 11036–11057 | | |
| Exon 11 | 5′-CCTCACCTCCTCCAGCTACTCC-3′ | 11163–11184 | 62 | 318 |
| | 5′-CAGAAGTTGGGGCTGAGACAGAG-3′ | 11458–11480 | | |
| Exon 12 | 5′-GCTCTATGGGCTCCTGGAAATG-3′ | 11529–11550 | 58 | 293 |
| | 5′-AAAGGGTCTTGGGGCAAGG-3′ | 11803–11821 | | |
| Exon 13 | 5′-CTGTCATGTCCCCCGCAC-3′ | 11765–11782 | 58 | 339 |
| | 5′-TGTCTCAGTCTTATACACAACCTCC-3′ | 12079–12103 | | |
| Exon 14 | 5′-TGGTGGTATTTTCCAGCCCAAG-3′ | 13484–13505 | 60 | 320 |
| | 5′-GCACTGAGGAATTTGGAGCGG-3′ | 13783–13803 | | |
| Exon 15 | 5′-AAGGCAGGAGGTGGGGAGTGACTG | 14045–14078 | 70 | 201 |
| | 5′-GGAAATGAGGACCTGGGGGGTATC | 14222–14245 | | |
| Exon 16 | 5′-TCCTGCTCCCACCCTTCCCC-3′ | 14673–14692 | 68 | 276 |
| | 5′-TCTGCCTCCCACCCTCCCAG-3′ | 14929–14948 | | |
| Exon 17 | 5′-TGGAGGAGGCAGGGGAGAAC-3′ | 15980–15999 | 70 | 347 |
| | 5′-GGGGCAGGAGGATGGTGAAG-3′ | 16307–16326 | | |
| Exon 18 | 5′-ATATGGTGCCTGTGTTTTATTCCC-3′ | 17705–17728 | 65 | 332 |
| | 5′-TGCCTATCACACCCCAGCAC-3′ | 18017–18036 | | |
| Exon 19 | 5′-GGTACAGGACCCTTTTCTGG-3′ | 17973–17992 | 60 | 334 |
| | 5′-GCCTGCCCTAGTTCTGAGAC-3′ | 18287–18306 | | |
| Exon 20 | 5′-TCTCACCCTGTCTCTCTCCTG-3′ | 18819–18839 | 65 | 198 |
| | 5′-GAGGTGCCCATGAACTTCTG-3′ | 18997–19016 | | |

six minutes, washed with deionized water, and soaked in deionized water for five minutes. It was then stained in 0.2% $AgNO_3$ solution for 20 minutes with gentle shaking, washed with deionized water, and soaked in deionized water for five minutes. The $AgNO_3$ solution and washing water were pooled and added with a few drops of HCl to convert $AgNO_3$ to AgCl before discarding. The gel was soaked in developer containing 3% $Na_2CO_3$ and 0.0185% formamide in deionized water for 4 to 10 minutes. When DNA bands were clearly observed, a solution of 10% citric acid in deionized water was immediately added into the developer to stop the staining reaction. The SSCP pattern on the gel was recorded into a computer by scanning with a scanner. The gel was also dried on a piece of filter paper for long-term storage.

Mobility shift of single strand DNA from the normal pattern indicated the presence of a possible mutation. The PCR product of the exon that showed mobility shift was analyzed by direct DNA sequencing.

*Direct DNA sequencing.* To identify mutation in the exons of *AE1* gene observed in the PCR-SSCP analysis, the PCR product was purified from a preparative agarose gel and sequenced by manual direct DNA sequencing using Thermo Sequenase Cycle Sequencing Kit (Amersham Life Science Inc., Arlington Heights, IL, USA) or by an automated sequencing machine (ABI-PRISM™ 310 Genetic Analyzer; ABI, USA) using an ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit.

## Red cell anion transport studies

$^{35}S$-$SO_4$ influx into red blood cells was measured in the two patients with SAO and dRTA and their family members, and normal controls in the presence and absence of the inhibitor, 4,4′-di-isothiocyanato-stilbene-2,2′-disulfonic acid (DIDS). The studies were performed at 37°C in buffer of 70 mM sodium citrate, 3 mM sodium sulfate, and 10 mM Tris, pH 7.4 [13].

**Table 2.** Renal acidification function after three days of $NH_4Cl$ loading in individuals with Southeast Asian ovalocytosis (SAO) compared with controls

| | SAO ($N = 10$) | Controls ($N = 4$) |
|---|---|---|
| Serum | | |
| Creatinine *mg/dL* | 0.9 ± 0.1 | 1.1 ± 0.1 |
| $K^+$ *mM* | 3.8 ± 0.1 | 3.7 ± 0.1 |
| $HCO_3^-$ *mM* | | |
| Pre-acid loading | 26 ± 0.8 | 27 ± 0.1 |
| Post-acid loading | 20 ± 0.7 | 20 ± 1.6 |
| Venous pH | | |
| Pre-acid loading | 7.35 ± 0.02 | 7.37 ± 0.03 |
| Post-acid loading | 7.32 ± 0.01 | 7.29 ± 0.02 |
| Urine | | |
| $C_{Cr}$ *ml/min* | 93 ± 6 | 87 ± 9 |
| pH | | |
| Pre-acid loading | 6.0 ± 0.1 | 6.0 ± 0.3 |
| Post-acid loading | 5.0 ± 0.1 | 4.9 ± 0.1 |
| After furosemide | 4.5 ± 0.1 | 4.4 ± 0.1 |
| $NH_4^+$ *μmol/min* | | |
| Pre-acid loading | 22 ± 0.3 | 23 ± 4 |
| Post-acid loading | 65 ± 6 | 65 ± 4 |
| After furosemide | 75 ± 6 | 63 ± 7 |

**Table 3.** Renal acidification after short acid loading in individuals with Southeast Asian ovalocytosis (SAO) compared with controls

| | SAO ($N = 10$) | Controls ($N = 7$) |
|---|---|---|
| Serum | | |
| Creatinine *mg/dL* | 1.1 ± 0.1 | 1.0 ± 0.2 |
| $K^+$ *mM* | 3.8 ± 0.04 | 3.9 ± 0.1 |
| $HCO_3^-$ *mM* | | |
| Pre-acid loading | 25 ± 0.7 | 25 ± 0.9 |
| Post-acid loading | 20 ± 0.6 | 22 ± 0.8 |
| Venous pH | | |
| Pre-acid loading | 7.37 ± 0.02 | 7.35 ± 0.01 |
| Post-acid loading | 7.31 ± 0.05 | 7.30 ± 0.02 |
| Urine | | |
| $C_{Cr}$ *ml/min* | 83 ± 6 | 90 ± 14 |
| pH | | |
| Pre-acid loading | 5.9 ± 0.1 | 5.6 ± 0.1 |
| Post-acid loading | 5.0 ± 0.1 | 4.9 ± 0.1 |
| $NH_4^+$ *μmol/min* | | |
| Pre-acid loading | 28.6 ± 3 | 21.5 ± 6 |
| Post-acid loading | 39.4 ± 6 | 36.7 ± 4 |

## RESULTS

### Clinical studies

Renal acidification was performed in 20 individuals with SAO and the 11 controls. The $NH_4^+$ excretion rate and urine pH after a three-day $NH_4Cl$ load are shown in Table 2. Neither subject groups (10 individuals vs. 4 controls) had a statistical significant difference in the $NH_4^+$ excretion rate (65 ± 6 vs. 65 ± 4 μmol/min) or urinary pH (5.0 ± 0.1 vs. 4.9 ± 0.1) following the acid load. After diuresis with oral furosemide on the fourth day of the acid load, the urinary pH decreased significantly in both groups, but there was no significant difference between the two groups (4.5 ± 0.1 vs. 4.4 ± 0.1). The increment of $NH_4^+$ excretion during the peak diuresis was not significantly different between the two groups (75 ± 6 vs. 63 ± 7 μmol/min). The maximum urine osmolality after 16 hours of water deprivation and intranasal DDAVP administration was also not significantly different between the two groups (940 ± 41 vs. 850 ± 46 mOsm/kg/$H_2O$).

Table 3 provides a summary of the results of urinary acidification studies after the short acid load. Blood pH values after the acid loading of 10 SAO and 7 control subjects were less than 7.35. Urine pH and $NH_4^+$ excretion rate after the acid load between both groups were not significantly different (5.0 ± 0.1 vs. 4.9 ± 0.1 and 39 ± 6 vs. 37 ± 4 μmol/min, respectively).

Pedigrees (KSN and YAT) of two subjects with SAO and dRTA are shown in Figure 1. Propositi (II-1 in both families) presented with history of growth retardation, SAO, and hypokalemia (Table 4). Complete dRTA was diagnosed by low $NH_4^+$ excretion rate in both propositi (3.3 and 4.3 μmol/min, respectively) as well as by the inability to lower the urine pH below 5.5 (7.3 and 6.7, respectively) in the presence of metabolic acidosis (venous pH 7.26 and 7.27 and serum $HCO_3^-$ 9 and 14 mEq/liter, respectively). No abnormal renal acidification was detected in both sets of parents (I-1 and I-2).

### Screening and characterization of AE1 gene mutations

Polymerase chain reaction-SSCP analysis was used to screen for mutations in exons 4 to 20 of the A*E1* gene and in intron 3, the promoter region of the kidney isoform. DNA samples from the propositi, siblings, as well as the parents of the two families were also analyzed. Figure 1 shows the results of PCR-SSCP analysis for exons 11 and 17 of one normal individual (N) and members of the two families. In the YAT family, the father (I-1) showed a mobility shift in exon 17, and the mother (I-2) showed a mobility shift in exon 11. In the KSN family, the father (I-1) demonstrated a mobility shift in exon 11, whereas the mother (I-2) showed a shift in exon 17. The DNA samples of both KSN and YAT families revealed mobility shifts in both exons 11 and 17. Except for a mobility shift of exon 4 (caused by the Memphis I polymorphism, confirmed by sequencing; Fig. 5), the PCR-SSCP patterns of all other exons, including intron 3, were normal (data not shown).

Because the mobility shifts of exons 11 and 17 of *AE1* gene detected by PCR-SSCP in the two patients were the same, amplified DNA of these two exons from the propositus of KSN family were sequenced. Exon 11 had a deletion of 27 bp corresponding to codons 400 to 408 (Fig. 2), whereas exon 17 contained a nucleotide substitution of G to A in codon 701 (CGG→CAG), resulting in an amino acid change from glycine to aspartic acid (G701D) (Fig. 3). Thus, the *AE1* gene of the propositi of

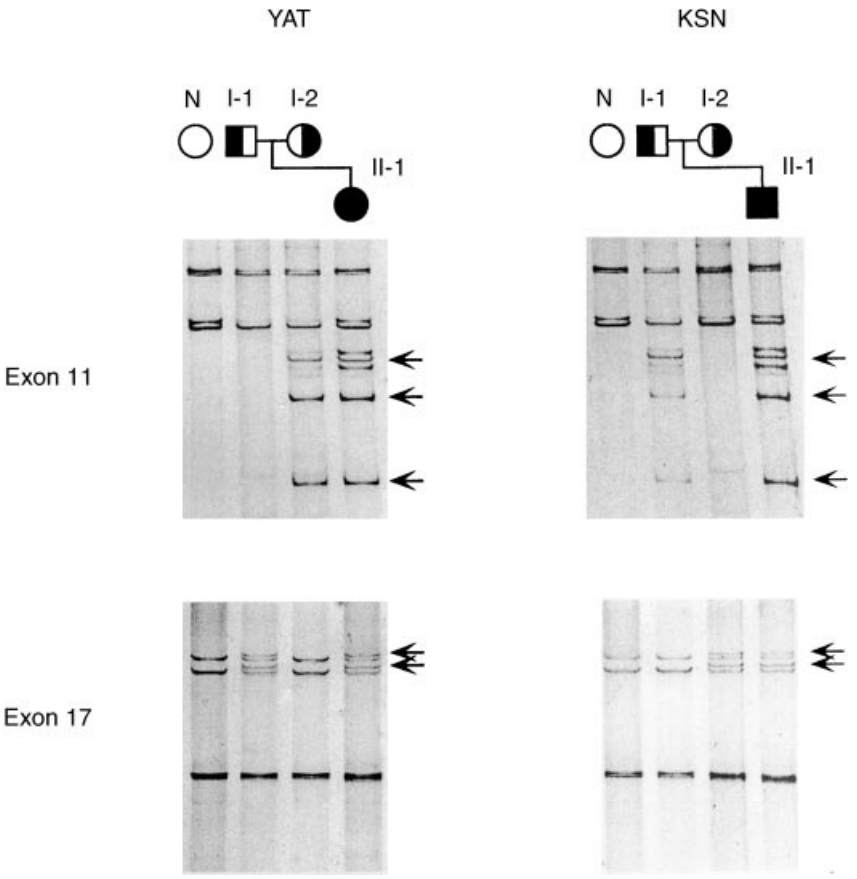


**Fig. 1. Screening of mutations in exons 11 and 17 of the *AE1* gene in the two patients with Southeast Asian ovalocytosis (SAO) and distal renal tubular acidosis (dRTA) and their parents by the PCR-SSCP technique.** The two patients (II-1 in both families) had mobility shifts of single-stranded DNAs in both exons 11 and 17 (arrows), whereas their parents had the mobility shifts in either exon 11 (I-2 in family of YAT and I-1 in family of KSN) or exon 17 (I-1 in family of YAT and I-2 in family of KSN). "N" is normal individual.

**Table 4.** Values in blood and urine collections from the two patients with SAO and dRTA

| | KSN: II–1 | YAT: II–1 |
|---|---|---|
| Serum | | |
| Creatinine *mg/dL* | 0.5 | 0.6 |
| BUN *mg/dL* | 13 | 13 |
| $Na^+$ *mM* | 137 | 141 |
| $K^+$ *mM* | 3.3 | 3.4 |
| $Cl^-$ *mM* | 116 | 111 |
| $HCO_3^-$ *mM* | 8.7 | 14 |
| Venous pH | 7.26 | 7.27 |
| Urine | | |
| TTKG | 10.4 | 10.3 |
| Flow rate *ml/min* | 0.4 | 0.8 |
| pH | 7.3 | 6.7 |
| $NH_4^+$ *μmol/min* | 3.3 | 4.3 |

Abbreviations are: SAO, Southeast Asian ovalocytosis; dRTA, distal renal tubular acidosis; BUN, blood urea nitrogen; TTKG, transtubular K concentration gradient.

both families was a compound heterozygosity of 27 bp deletion in exon 11 and missense mutation (CGG→CAG) in exon 17. The sequencing results also showed the presence of homozygous band 3 Memphis I (Fig. 5) in the two patients. The presence of exon 11 deletion and exon 17 missense mutation in both patients was also confirmed by the detection of a shorter PCR product and elimination of *HpaII* restriction site on the amplified DNA, respectively, by gel electrophoresis (Fig. 4). Mutations of *AE1* gene were also analyzed by PCR-SSCP method in 20 individuals with SAO and in normal subjects. SAO individuals had mobility shifts in exons 4 and 11.

### Anion transport property of the red cells of the two families

An influx of [$^{35}$S] sulfate into the red cells of members of the two families was compared with that of red cells from 10 normal controls taken at the same time. Red cell samples from the propositi of the KSN and YAT families and family members with SAO showed a consistently lower anion transport activity than the normal samples in both the presence and absence of DIDS (Table 5). Family members with only the exon 17 mutation had normal anion transport and DIDS activity.

## DISCUSSION

Mutation of the *AE1* gene in SAO has been the subject of a number of studies [1, 4, 27]. The underlying molecular defect is a 27 bp deletion in exon 11 of the *AE1* gene, resulting in the loss of 9 amino acids (codons 400 to 408) in the band 3 protein, which is also associated with the

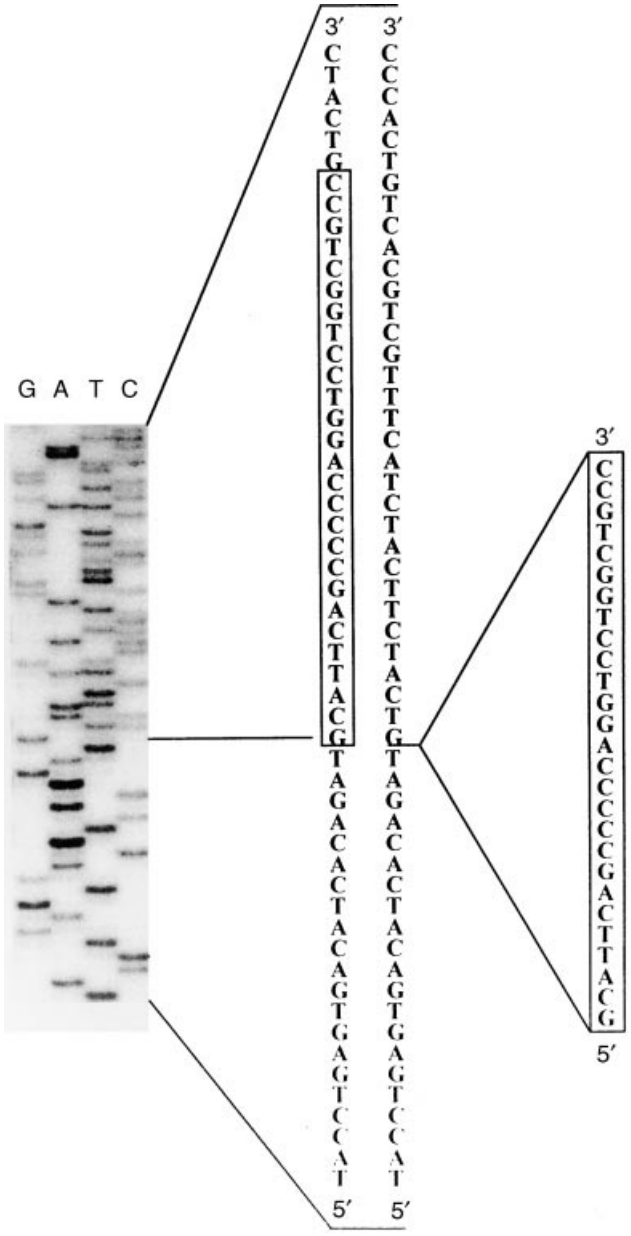


**Fig. 2. Sequencing analysis of exon 11 of the *AE1* gene in the patient with dRTA and SAO (II-1, family of KSN), showing nucleotide sequence of exon 11 with 27 nucleotide deletion superimposing the normal sequence.** Identical 27 nucleotides in the normal and deleted alleles (the latter is separated from the nucleotide stretch) are blocked. The deletion of 27 nucleotides in one allele resulted in shifting of the remaining nucleotide sequence superimposing the normal sequence in the autoradiogram.

Memphis I (K56E) polymorphism. Although SAO occurs with high frequency in parts of Southeast Asia and Melanesia, no homozygous individual for the *AE1* mutation has been identified, suggesting that homozygosity for this mutation may be lethal [1].

Anion exchanger 1 in red cells is important for the transport of carbon dioxide from the tissue to the lung and for acid secretion in type A intercalated cells of the kidney [28]. Total deficiency of red cell band 3 caused by a nonsense mutation has been reported in cattle [29]. Animals showed a moderate uncompensated anemia with hereditary spherocytosis and retarded growth, which was attributed to mild acidosis. The band 3-deficient animals had defective renal acid secretion and could not acidify urine pH below 7.5 despite metabolic acidosis. In dRTA, acid secretion in the distal nephron is impaired, leading to the development of metabolic acidosis [10, 30]. Recently, several studies have demonstrated associations of the *AE1* mutations and dRTA: Rysava et al reported that 2 out of 10 patients with hereditary spherocytosis and band 3 PRIBRAM (G→A in the first nucleotide of intron 12) had an incomplete form of dRTA [14]; Bruce et al reported an association between familial dRTA and point mutations of the *AE1* gene, namely, R589H, R589C, and S613F [12]. The *AE1* mutation, R589H, has also been reported in two other studies [13, 31]. An intragenic 13 bp duplication resulting in deletion of the last 11 amino acids of *AE1* gene in one dRTA subject has also been demonstrated [31]. Mutations in the *AE1* gene appear to cause autosomal dominant dRTA [12, 13, 31], but the molecular mechanism is unknown.

There have been two previous studies showing the association between dRTA and elliptocytosis or SAO [15, 16]. The presence of the two conditions in the same individuals suggests that there may be a common underlying molecular defect. However, mutation of the *AE1* gene in individuals with both of these conditions was not demonstrated. In this study, 20 individuals with SAO, confirmed by the presence of 27 bp deletion in exon 11 of the *AE1* gene, showed no abnormal renal acidification following the three-day $NH_4Cl$ loading ($N = 10$; Table 2) or by short acid loading ($N = 10$; Table 3). The rate of excretion of $NH_4^+$ increased by almost threefold, and the urine pH decreased below 5.0 after three-day acid loading and during the furosemide-induced diuresis to typical values of normal subjects [19]. This suggests that the rate of production of $NH_4^+$ in the proximal tubular cells was not appreciably depressed. Thus, SAO mutation of the *AE1* gene in the heterozygous condition is not sufficient to cause dRTA.

The two clinically affected unrelated patients in the KSN and YAT families with dRTA and SAO showed a low rate of $NH_4^+$ excretion and an inability to lower the urine pH below 5.5 in the presence of systemic acidosis (Table 4). There was also a high transtubular $[K^+]$ gradient (TTKG) [25] given the degree of hypokalemia. No abnormal renal acidification was detected in either set of parents. Analysis of *AE1* gene mutation by PCR-SSCP method showed that the two patients (II-1 in both families) had the same mobility shifts in exons 11 and 17, whereas the parents had mobility shift in either exon

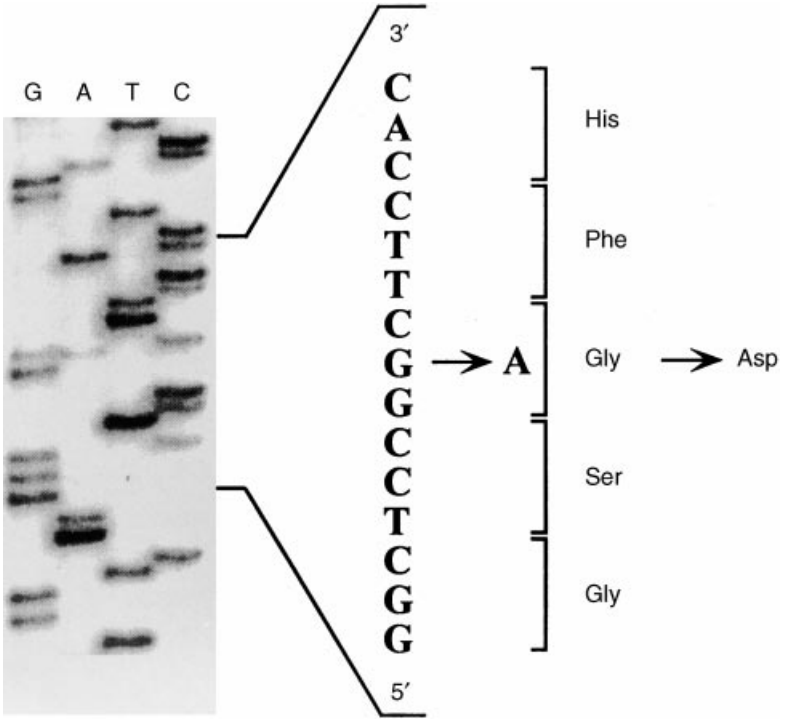


**Fig. 3. Sequencing analysis of exon 17 of *AE1* gene in the patient with SAO and dRTA (II-1, family of KSN).** A substitution from G to A in the second nucleotide of codon 701 was observed. This substitution results in missense mutation changing the amino acid at the position 701 from glycine to aspartic acid (G701D).

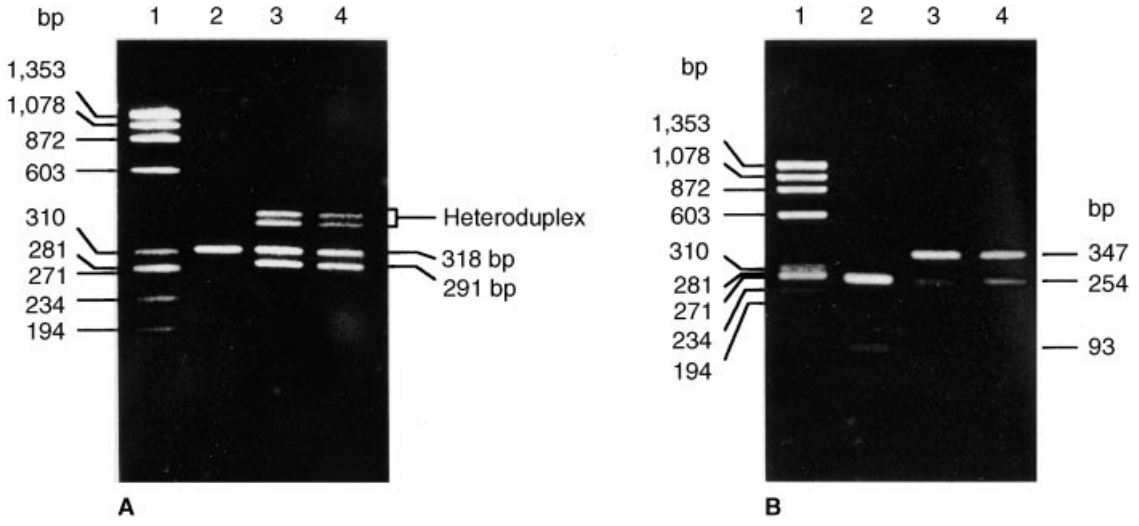


**Fig. 4. Detection of exon 11 deletion and exon 17 (CGG→CAG) missense mutation in the patients (II-1) of KSN and YAT families by agarose gel electrophoresis.** (*A*) PCR products from amplifications of exon 11 of *AE1* gene with AE1Ex11L/AE1Ex11R primers. Normal control sample (lane 2) showed only a PCR product with the size of 318 bp. DNA samples from the patients of the KSN (lane 3) and YAT (lane 4) families who had 27 bp deletion in exon 11 in one allele of the *AE1* genes resulted in PCR products with the sizes of 318 and 291 bp, and also their heteroduplexes. (*B*) PCR products from amplifications of exon 17 with AE1Ex17L/AE1Ex17R primers and digestions with *Hpa*II restriction endonuclease, which could digest the normal (CCGG) but not the mutant (CCAG) sequences. A normal control sample (lane 2) showed digested fragments with the sizes of 254 and 93 bp. DNA samples from the patients of the KSN (lane 3) and YAT (lane 4) families who had exon 17 missense mutation in one allele of the *AE1* gene revealed both digested (254 and 93 bp) and undigested (347 bp) PCR products. Lane 1 in both sets is Phix174 DNA/*Hae*III markers.

11 (I-2 in family YAT and I-1 in family KSN) or exon 17 (I-1 in family YAT and I-2 in family KSN; Fig. 1). DNA sequencing (Figs. 2 and 3) and gel electrophoresis (Fig. 4) revealed that exon 11 contained a 27 bp deletion typical of SAO [4] and exon 17 had a single nucleotide substitution of G to A in the second nucleotide of codon 701 (CGG→CAG), resulting in a G701D missense mutation. This is the first report of a compound heterozygosity

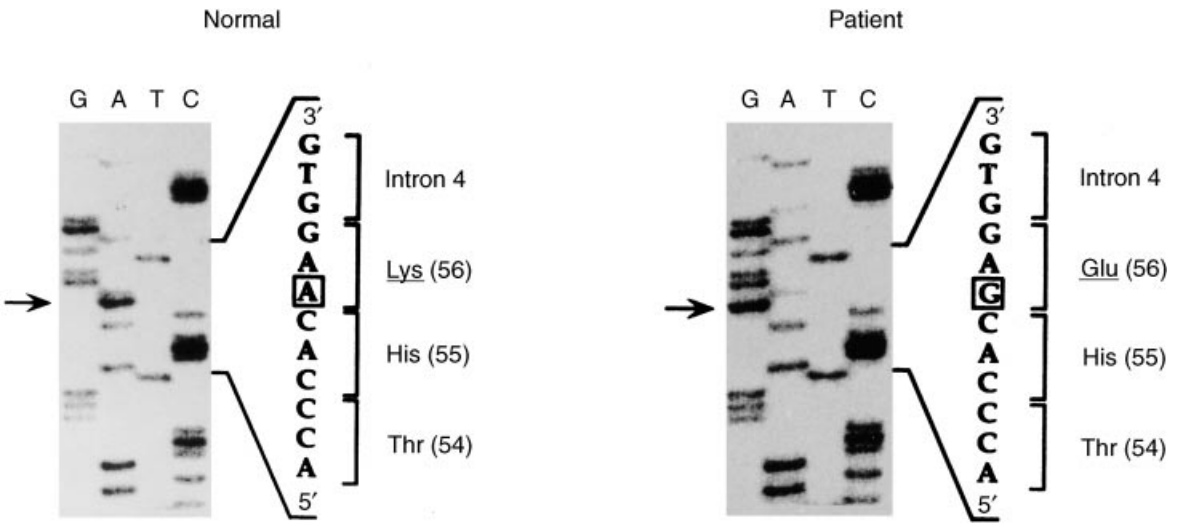


**Fig. 5. Sequencing analysis of exon 4 of the *AE1* gene in a normal individual and the patient with SAO and dRTA (II-1, the family of KSN).** The normal individual had AAG (code for lysine), but the patient had GAG (code for glutamic acid) at codon 56. The band 3 variant that had glutamic acid at the position 56 was previously described as band 3 Memphis I. The patient who had the 27 bp deletion in exon 11 and the G→A substitution in codon 701 in exon 17 was homozygous for band 3 Memphis I. Therefore, these two mutations were linked to band 3 Memphis I polymorphism.

**Table 5.** Characterization of $[^{35}S]SO_4$ influx into red cells from members of families of YAT and KSN, compared with healthy control cells

| | | | $SO_4$ uptake *10–17 mol/min/cell* | | |
|---|---|---|---|---|---|
| | Genotype | Phenotype | No DIDS | 1.5 μM DIDS | % |
| YAT family | | | | | |
| I–1 | G701D | | 6.45 | 2.12 | 32.9 |
| I–2 | Ex 11Δ27 | SAO | 4.78 | 0.08 | 1.7 |
| II–1 | Ex11Δ27/G701D | SAO + RTA | 4.77 | 0.13 | 2.7 |
| KSN family | | | | | |
| I–1 | Ex 11Δ27 | SAO | 3.57 | 1.48 | 24.1 |
| I–2 | G701D | | 6.56 | 2.35 | 35.8 |
| II–1 | Ex11Δ27/G701D | SAO + RTA | 4.03 | 0.15 | 3.7 |
| Controls | | | 6.0 ± 0.8 | | |

for *AE1* mutations associated with SAO and dRTA, to our knowledge. The sequencing results also showed the presence of homozygous band 3 Memphis I (Fig. 5) in the two patients, indicating that the two mutations linked with band 3 Memphis I polymorphism. Studies of the influx of $[^{35}S]$ sulfate into red cells of the two patients and their family members with SAO indicated a consistently lower anion transport activity than the normal red cells (Table 5). Individuals with exon 17 mutation had normal red cell anion transport activity.

Glycine 701 is located at the beginning of membrane span 9 of band 3, which is in a highly conserved region in the AE protein family across several species [28], which indicates its structural or functional importance. Although the presence of both SAO and G701D mutations had no additional affect on the influx of $[^{35}S]$ sulfate into red cells than the presence of the SAO mutation alone, the $HCO_3^-/Cl^-$ anion exchanger activity in the type A intercalated cells of the renal collecting duct in individuals with the combined mutations may be abnormal. Two siblings with dRTA and hemolytic anemia have recently been found to carry a homozygous G701D missense mutation of the *AE1* gene, which causes recessively transmitted dRTA in this kindred with apparently normal erythroid anion transport in the parents and affected children [32].

An expression study of the G701D mutation in *Xenopus* oocytes has shown that the mutant protein was not transported to the surface of the cell membrane [32]. However, when it was co-expressed with glycophorin A, the erythroid band 3 chaperonin, both AE1 surface expression and AE1-mediated $Cl^-$ transport were rescued. This suggests that the G701D mutation may lead to decreased or absent AE1 accumulation at the basolateral membrane of the type A intercalated cells in the collecting duct. Therefore, the presence of both SAO and G701D mutations would have a greater effect to the type A intercalated cells than the presence of either mutation alone,

and this would explain the abnormal urinary acidification in the patients with the compound heterozygosity.

Although mutations of the *AE1* gene have been reported to be associated with autosomal dominant dRTA [12, 13, 31], the presence of the compound heterozygosity of *AE1* mutations associated with SAO and dRTA shown in our study, and of homozygosity of AE1 mutation associated with dRTA and hemolytic anemia in that of Tanphaichitr et al [32] indicate an autosomal recessive mode of inheritance.

## ACKNOWLEDGMENTS

This study has been supported by grants from Research Development and Medical Education Fund of the Faculty of Medicine, Siriraj Hospital. P. Malasit and P. Wilairat are recipients of the Senior Research Grant of The Thailand Research Fund (TRF). The Medical Molecular Biology Laboratory also operates as a Medical Biotechnology Unit of the National Center for Genetic Engineering (BIOTEC) of the National Science and Technology Development Agency (NSTDA), Thailand. We thank Miss Duangporn Chuawatana, Sumitra Mingkum, and Nunghathai Sawasdee for their technical assistance.

*Reprint requests to Somkiat Vasuvattakul, M.D., Renal Unit, Department of Medicine, Siriraj Hospital, Mahidol University, Bangkok 10100, Thailand.*
*E-mail: sisva@mucc.mahidol.ac.th*

## REFERENCES

1. Liu S, Zhai S, Palek J, Golan DE, Amato D, Hassan K, Nurse GT, Babona D, Coetzer T, Jarolim P, Zaik M, Borwein S: Molecular defect of the band 3 protein in Southeast Asian ovalocytosis. *N Engl J Med* 323:1530–1538, 1990
2. Jones GL, Edmundson HM, Wesche D, Saul A: Human erythrocyte band 3 has an altered N-terminus in malaria resistant Melanesian ovalocytosis. *Biochim Biophys Acta* 1096:33–40, 1991
3. Schofield AE, Reardon DM, Tanner MJA: Defective anion transport activity of the abnormal band 3 in hereditary ovalocytic red blood cells. *Nature* 355:836–838, 1992
4. Jarolim P, Palek J, Amato D, Hassan K, Sapak P, Nursel GT, Rubin HL, Zhai S, Sahr KE, Liu SC: Deletion in erythrocyte band 3 gene in malaria-resistant Southeast Asian ovalocytosis. *Proc Natl Acad Sci USA* 88:11022–11026, 1991
5. Alper SL, Natale J, Gluck S, Lodish HF, Brown D: Subtypes of intercalated cells in rat kidney collecting duct defined by antibodies against erythroid band 3 and renal vacuolar $H^+$ ATPase. *Proc Natl Acad Sci USA* 86:5429–5433, 1989
6. Wagner S, Vogel R, Lietzke R, Koob R, Drenckhahn D: Immunochemical characterization of a band 3-like anion exchanger in collecting duct of human kidney. *Am J Physiol* 253:213–221, 1987
7. Wainwright SD, Tanner MJA, Martin GEM, Yendle JE, Holmes C: Monoclonal antibodies to the membrane domain of the human erythrocyte anion transport protein. *Biochem J* 258:211–220, 1989
8. Kollerton-Jons A, Wagner S, Hubner S, Appelhans H, Drenckhahn D: Anion exchanger 1 in human kidney and oncocytoma differs from erythroid AE1 in its NH2 terminus. *Am J Physiol* 265:F813–F821, 1993
9. Schofield AE, Martin PG, Spillett D, Tanner MJA: The structure of the human red blood cell anion exchanger (EPB3, AE1, band 3) gene. *Blood* 84:2000–2012, 1994
10. Wrong O, Unwin R, Cohen E, Tanner M, Thakker R: Unraveling the molecular mechanisms of kidney stones. *Lancet* 348:1561–1565, 1996
11. Batlle D, Flores G: Underlying defects in distal renal tubular acidosis: New understandings. *Am J Kidney Dis* 27:896–915, 1996
12. Bruce LJ, Cope DL, Jones JK, Schofield AE, Burley M, Povey S, Unwin RJ, Wrong O, Tanner MJA: Familial distal renal tubular acidosis is associated with mutations in the red cell anion exchanger (band 3, AE1) gene. *J Clin Invest* 100:1693–1707, 1997
13. Jarolim P, Shayakul C, Prabakaran D, Jiang L, Stuart-Tilley A, Rubin H, Simova S, Zavadil J, Herrin JT, Brouillette J, Somers MJG, Seemanova E, Brugnara C, Guay-Woodford LM, Alper SL: Autosomal dominant distal renal tubular acidosis is associated in three families with heterozygosity for the R589H mutation in the AE1 (band 3) $Cl^-/HCO_3^-$ exchanger. *J Biol Chem* 273:6380–6388, 1998
14. Rysava R, Tesar V, Jirsa M, Brabec V, Jarolim P: Incomplete distal renal tubular acidosis coinherited with a mutation in the band 3 (AE1) gene. *Nephrol Dial Transplant* 12:1869–1873, 1997
15. Thong MK, Tan AAL, Lin HP: Distal renal tubular acidosis and hereditary elliptocytosis in a single family. *Singapore Med J* 38:388–390, 1997
16. Baehner RL, Gilchrist GS, Anderson EJ: Hereditary elliptocytosis and primary renal tubular acidosis in a single family. *Am J Dis Child* 115:414–419, 1968
17. Lie-Injo LE, Fix A, Bolton JM, Gilman RH: Haemoglobin E-hereditary elliptocytosis in Malayan aborigines. *Acta Haematol* 47: 210–216, 1972
18. Wrong O, Davies H: The excretion of acid in renal disease. *Q J Med* 28:259–313, 1959
19. Vasuvattakul S, Gougoux A, Halperin ML: A method to evaluate renal ammoniagenesis in vivo. *Clin Invest Med* 16:265–273, 1993
20. Vasuvattakul S, Nimmannit S, Chaovakul VWS, Shayakul C, Malasit P: The spectrum of endemic renal tubular acidosis in the northeast of Thailand. *Nephron* 74:541–547, 1996
21. Van Slyke DD, Linder GS, Hiller A, Letter L, McIntosh JF: The excretion of ammonia and titrable acid in nephritis. *J Clin Invest* 2:255–288, 1926
22. Hastings AB, Sendroy J: The effect of variation in ionic strength on the apparent first and second dissociation constants of carbonic acid. *J Biol Chem* 65:445–455, 1925
23. Halperin ML, Vinay P, Gougoux A, Pichette C, Jungus RL: Regulation of the maximum rate of renal ammoniagenesis in the acidotic dog. *Am J Physiol* 248:F607–F615, 1985
24. Camara AA, Arn KD, Reimer A, Newburgh LH: The twenty-four hourly endogenous creatinine clearance as a clinical measure of the functional state of the kidneys. *J Lab Clin Med* 37:743–763, 1951
25. Ethier J, Kamel K, Magner P, Lemann JJ, Halperin ML: The transtubular potassium concentration in patients with hypokalemia and hyperkalemia. *Am J Kidney Dis* 15:309–315, 1990
26. Grunebaum L, Cazenave J-P, Camerino G, Kloepfer C, Mandel J-L, Tolstoshev P, Jaye M, De la Shalle H, Lecocq J-P: Carrier detection of hemophilia B by using a restriction site polymorphism associated with the coagulation factor IX gene. *J Clin Invest* 73:1491–1495, 1984
27. Tanner MJA: Molecular and cellular biology of the erythrocyte anion exchanger (AE1). *Semin Hematol* 30:34–57, 1993
28. Tanner MJA: The structure and function of band 3 (AE1): Recent developments. (review) *Mol Membr Biol* 14:155–165, 1997
29. Inaba M, Yawata A, Koshino I, Sato K, Takeuchi M, Takakuwa Y, Manno S, Yawata Y, Kanzaki A, Ji S, Ban A, Ki O, Maede Y: Defective anion transport and marked spherocytosis with membrane instability caused by hereditary total deficiency of red cell band 3 in cattle due to a nonsense mutation. *J Clin Invest* 97:1804–1817, 1996
30. Morris RC, Ives HE: Inherited disorders of the renal tubule, in *The Kidney*, edited by Brenner BM, Philadelphia, W. B. Saunders, 1996, pp 1764–1827
31. Karet FE, Gainza FJ, Gyory AZ, Unwin RJ, Wrong O, Tanner MJA, Nayir A, Alpay H, Santos F, Hulton SA, Bakkaloglu A, Ozen S, Cunningham MJ, Di Pietro A, Walker WG, Lifton RP: Mutations in the chloride-bicarbonate exchanger gene AE1 cause autosomal dominant but not autosomal recessive distal renal tubular acidosis. *Proc Natl Acad Sci USA* 95:6337–6342, 1998
32. Tanphaichitr VS, Sumboonnanonda A, Ideguchi H, Shayakul C, Brugnara C, Takao M, Veerakul G, Alper SL: Novel AE1 mutations in distal renal tubular acidosis: Loss-of-function rescued by glycophorin A. *J Clin Invest* 102:2173–2179, 1998